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Rahim, Abdoul; Peters, Günther H.J.; Jalkanen, Karl J.; Westh, P

Published in:
Current Physical Chemistry

Publication date:
2013

Document Version
Early version, also known as pre-print

[Link back to DTU Orbit](#)

Citation (APA):
Rahim, A., Peters, G. H. J., Jalkanen, K. J., & Westh, P. (2013). Effects of mannose, fructose, and fucose on the structure, stability, and hydration of lysozyme in aqueous solution. *Current Physical Chemistry*, 3(1), 113-125.

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Effects of mannose, fructose, and fucose on the structure, stability, and hydration of lysozyme in aqueous solution

Abdoul Rahim,^{1,*} G.H. Peters,² K.J. Jalkanen,^{3,*} and P. Westh¹

¹Roskilde University, Department of Science, Systems and Models, Universitetsvej 1, Postboks 260, DK-4000 Roskilde, Denmark; Tel1: +45 4242 5484 (AR); Tel2: +45 4674 2879 (PW);

Fax: +45 4674 3011

*E-mail: arahim@ruc.dk (corresponding author); Email: pwesth@ruc.dk

Abdoul Rahim url: <http://www.researcherid.com/rid/B-8760-2012>

P. Westh Lab url: <http://www.ruc.dk/~pwesth>

²Technical University of Denmark, DTU Chemistry, Department of Chemistry, Kemitorvet, DK-2800 Kgs. Lyngby, Denmark

Email: ghp@kemi.dtu.dk

G.H.J. Peter Lab url: <http://www.biofysisk.kemi.dtu.dk/ki-2636.aspx>

³Technical University of Denmark, DTU Nanotech, Department of Micro- and Nanotechnology, Ørsted Plads, DK-2800 Kgs. Lyngby, Denmark

*Email: karljalkanen@gmail.com (corresponding author)

DTU Nanotech Lab url: <http://www.nanotech.dtu.dk/Research/Research%20groups/TheoBio.aspx>

K.J. Jalkanen url: <http://www.researcherid.com/rid/A-2456-2008>

Abstract

The bio-protective properties of monosaccharides, namely mannose, fructose and fucose, on the stability and dynamical properties of the NMR determined hen egg-white lysozyme structure have been investigated by means of molecular dynamics simulations at room temperature in aqueous solution and in 7 and 13 wt % concentrations of the three sugars. Results are discussed in the framework of the bio-protective phenomena. The three sugars show similar bio-protective behaviours at room temperature (300 K) in the concentration range studied as shown by the small RMSDs of the resulting MD structures from that of starting NMR structure. The effects of sugars on protein conformation are found to be relatively strong in that the conformation of lysozyme is stable after an initial 9 ns equilibration for fucose and mannose and 12 ns equilibration for fructose, respectively, at high concentrations. For mannose the final RMSD is significantly smaller than that of fucose and fructose at the higher concentration, while at the lower concentration the RMSD are essentially the same. The radial distribution function of the water and sugars around lysozyme was used to monitor the preferential hydration. Analysis of the solvent and sugar distributions around lysozyme was used to investigate the interfacial solvent and sugar structure near the protein surface.

Keywords: Osmolytes, molecular dynamics, lysozyme, preferential hydration

Introduction

The tendencies of proteins to decompose denature, and aggregate are critical problems in the biotechnology, food, cosmetics, and pharmaceutical industries. The chemical (oxidation, deamidation, and hydrolysis) and physical (unfolding and aggregation) changes of proteins during the formulation process, and storage not only reduce biological activity, but they can also cause adverse reactions [1]. In order to protect proteins against denaturation and aggregation, there have been efforts at various levels, which include chemical modification of proteins and controlling the protein environment using co-solvents. To perform their function all of these substances, generally known as co-solvents or osmolytes like sugars, amino acids, salts, polyols, and nucleic acids, must be used at relatively high concentrations. Typically, concentrations of osmolytes as high as 20 wt % of dry weight are needed to efficiently protect proteins both in solution and in the dry state during and after lyophilization and in organisms capable of surviving complete dehydration [2,3]. Initially urea was also used as a cosolvent to solubilize proteins which were not soluble in aqueous solution [4,5,6], but subsequently it was found to denature some proteins when used at high concentrations [7].

The ability of carbohydrates to stabilize proteins has been attributed to the preferential hydration of protein that occurs when it is dissolved in a sugar solution [8,9,10,11]. This phenomenon is due to the fact that carbohydrates, in general, do not interact directly with the protein molecules, and are therefore called non-perturbing osmolytes. There are cases though, especially during drying and freezing in the lyophilization process, that carbohydrate molecules do directly interact via hydrogen bonding with protein molecules [3]. An increase in the concentration of carbohydrate in bulk water engages more water molecules in solubilization and at high carbohydrate concentrations protein and solutes begin to compete for the available water. This competition leads to reduction in the protein solvation layer, which results in a decrease in the apparent molal volume of the protein. As a result, the protein becomes more compact and less susceptible to destabilizing forces such as high temperature and the presence of destabilizing osmolytes or agents like urea [8-11].

Among several osmolytes, sugars have been known to stabilize the protein conformation against chemical denaturation or reaction, thermal denaturation, and loss of their biological activity, which can be caused by an increase in temperature, a change in pH value and the addition of various chemicals [12]. Sugars belong to a class of osmolytes that in nature are synthesized to protect organisms against the stresses of high osmotic pressure and freezing, and sugar synthesis is a good example of a defensive reaction of many organisms. Sugars are commonly employed in freeze-drying formulations of therapeutic proteins to preserve their activity [13].

The properties and mechanism of the sugar bio-protective phenomenon are still under discussion. Several hypotheses have been proposed, but none of them can be considered as fully accepted. They mainly relate to the following properties: (i) sugar are able to replace more water molecules, stabilizing the 3D protein structure during dehydration, and create sugar-protein hydrogen bonds replacing the water protein hydrogen bonds [14], (ii) sugars destructure the hydrogen bond network of water molecules and prevent the formation of ice because sugars bind to a number of water molecules and therefore have a destructuring effect [15], (iii) sugars are co-solvents thus inducing greater thermodynamic stabilization (perhaps protect protein from conformational

disorders) [16], and (iv) sugars possess a higher glass transition temperature, which results in a stronger decrease of the protein dynamical fluctuations [17].

Numerous experimental works and molecular dynamics simulations have been performed on the structure and dynamics of ternary protein, sugar and water solutions [18] and more generally the stability of lysozyme [19]. Sola-Penna and co-workers showed by fluorescence measurements that the sugars (trehalose, glucose, fructose and sucrose) protect the function and the structure of cytosolic enzyme against chemical denaturation. They suggested that the effectiveness of sugar or carbohydrates for protection of enzymes depends on the nature of the sugar and of the enzyme [20]. Khan and Shabnum investigated the stabilization effect of sugar (fructose, sucrose, maltose and glucose) on the thermal and chemical denaturation of rabbit serum albumin by viscosity and far UV circular dichroism measurements. They showed that the stabilizing effect of the sugars seems to occur by the preferential exclusion of the respective sugar from the surface of the protein [21]. Rösger *et al.* have studied the thermodynamically nonideal behavior in crowded osmolyte solutions. Using Kirkwood-buff theory, they calculated the molar activity coefficients for the protective (stabilizing) osmolytes, mannose and fucose, and the denaturing (destabilizing) osmolyte, urea. They showed that the protective osmolytes have a much more concentration-dependent protein solvation behavior than do denaturing osmolytes [22]. Ionov and Hedoux investigated the bioprotective properties of disaccharides trehalose, sucrose and maltose on lysozyme using Raman spectroscopy and molecular dynamics simulation. They showed that sugars preserve the native state of the protein and shift the denaturation temperature to higher temperatures [23]. Drima and co-workers have studied lysozyme in a sugar glass at different temperatures and showed that a strong coupling between lysozyme and sugar molecules correlated with the dynamics of protein-sugar intermolecular hydrogen bonds (HBs) [24].

In order to better understand the physico-chemical properties of sugar, in the framework of the bio- protective solvent stability and to understand how globular proteins in their flexible native state at room temperature are influenced by solutes at intermediate concentrations, molecular dynamics simulations of the egg-white lysozyme in presence of solutions of three monosaccharides mannose, fructose and fucose in the 7-13 wt % concentration range have been performed.

The lysozyme-sugar and lysozyme-water interaction will be responsible for the specific effects for a given protein. The HBs capabilities of fructose and mannose are directly comparable since they possess the same chemical formula $C_6H_{12}O_6$ and the same number of hydroxyl groups. The lysozyme-fucose and lysozyme-water interactions and their HBs should be different than those with fructose and mannose, because fucose has a smaller number of oxygen ether and hydroxyl groups. Fructose forms similar numbers of HBs with lysozyme as does mannose, while fucose forms significantly fewer HBs with lysozyme at the studied concentrations.

From the preferential hydration hypothesis [11], this work confirms that sugars are good protein stabilizers. We have characterized the relative local distribution of water molecules around lysozyme in a similar way to that done by Cottone *et al.* [25]. We have used the relative local distribution of water and sugar molecules around lysozyme as a measure of the preferential hydration of lysozyme with mannose, fructose and fucose. Lysozyme clearly appears to be more preferentially hydrated with increasing sugar concentration.

Methods

Molecular Dynamics Simulations Details

Hen egg-white lysozyme (hereafter referred to as lysozyme) is a small globular protein consisting of 129 amino acids residues. The starting structure of Lysozyme (molecular mass of approximately 14.5 kDa) was obtained from the NMR solution structure deposited in protein data bank (PDB) (1e8L) [26]. Most probable charge states at pH 7 were chosen for ionizable residues. The total charge (+8) on lysozyme was neutralized by adding eight Cl^- ions. The solution concentrations of sugars on a protein-free basis were 13 and 7 wt % respectively. Lysozyme and its 5537 TIP3 water molecules were placed in a box with cell parameters $a = 60.50 \text{ \AA}$, $b = 57.13 \text{ \AA}$ and $c = 52.64 \text{ \AA}$. Then, monosaccharide molecules were located around lysozyme to ensure an isotropic distribution of sugars. Molecular dynamics simulations of lysozyme in sugar and water solution have been performed using the molecular dynamics program NAMD2 [27]. The all-atom CHARMM22 force field has been used for protein and sugar [28]. Visualization was done by using the VMD1.8.6 package [29].

The molecular dynamics simulations were carried out with a constant number of particles (N), pressure (P) and temperature (T), i.e., NPT ensemble. The long range electrostatic interactions were treated with the particle mesh Ewald [30]. The velocity Verlet algorithm with a 1 fs step size was employed to integrate the equations of motion. A Lennard-Jones potential has been employed to represent the van der Waals interaction. A cutoff radius of 12 \AA has been used for the long range electrostatic interactions, which were switched to zero between 10 and 14 \AA . Periodic boundary conditions were employed in the x, y and z directions. Temperature and pressure were maintained at 300 K and 1 atm, respectively, using the Langevin thermostat with a damping constant of 5 ps^{-1} , and the Langevin piston with a period of 200 fs and a decay constant of 500 fs. Coordinates were saved every 500 fs. The whole system was energy minimized for 3000 steps. Simulations were carried out for 30 ns for lysozyme in the sugar solutions and for 70 ns for lysozyme in the water solution. A simulation of lysozyme in pure water was performed under the same conditions as the mixtures. Table 1 summarizes some simulation data for the different systems considered in the present study.

Wt (%)	$N_{\text{lysozyme}}/N_{\text{sugar}}/N_{\text{water}}$	Density (g.cm^{-3})			Eq./Sim. Time (ns)		
		lysozyme			lysozyme		
0	1/0/5537	1.04			2/70		
		Fucose	fructose	mannose	Fucose	fructose	mannose
7	1/40/5217	1.04	1.05	1.05	2/30	2/30	2/30
13	1/81/4889	1.05	1.06	1.06	2/30	2/30	2/30

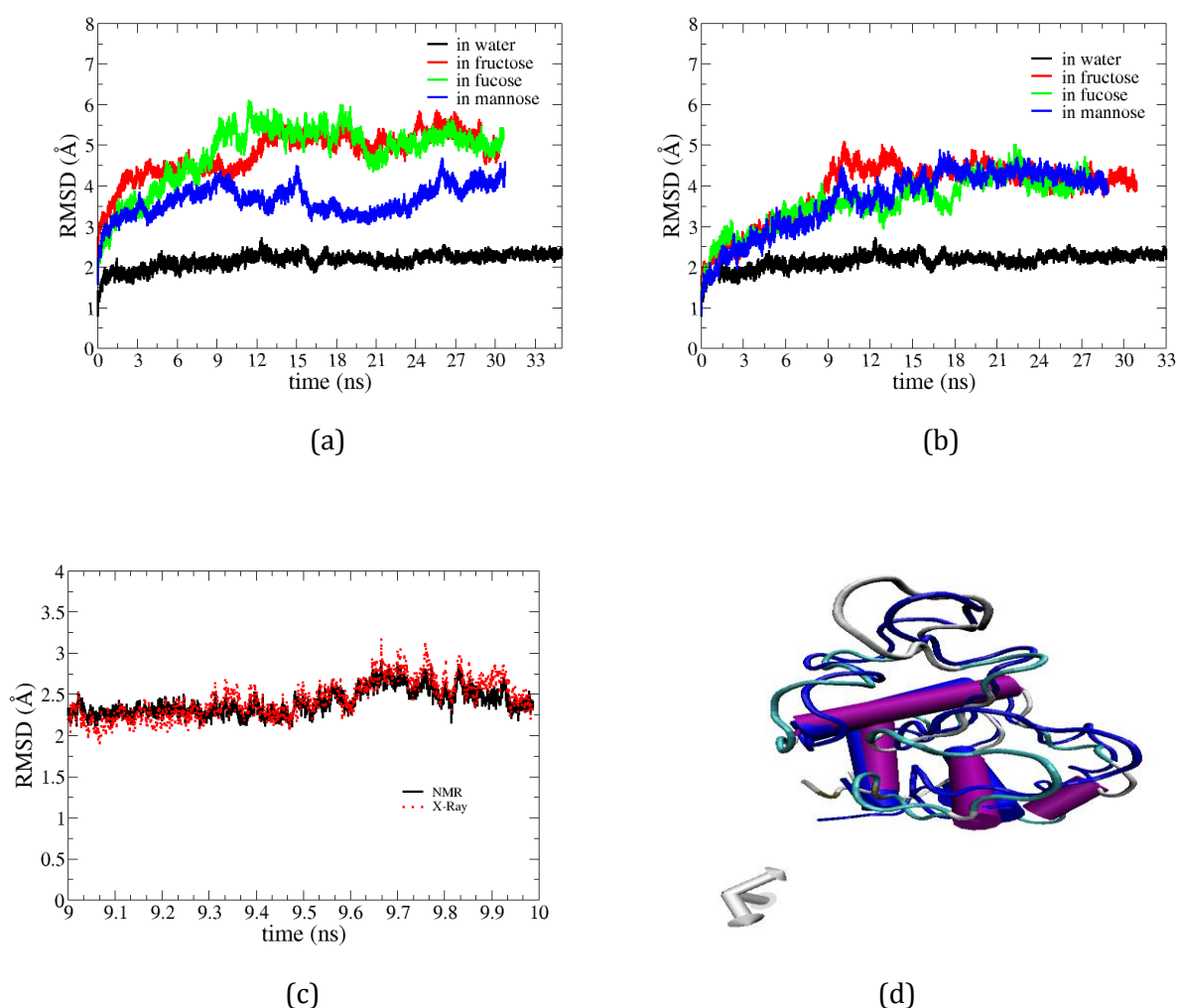
Table 1: System compositions (where N_{lysozyme} , N_{sugar} and N_{water} denote the number of lysozyme sugar and water molecules, respectively), densities and equilibration/simulation times for the different sugar concentrations on a protein-free basis.

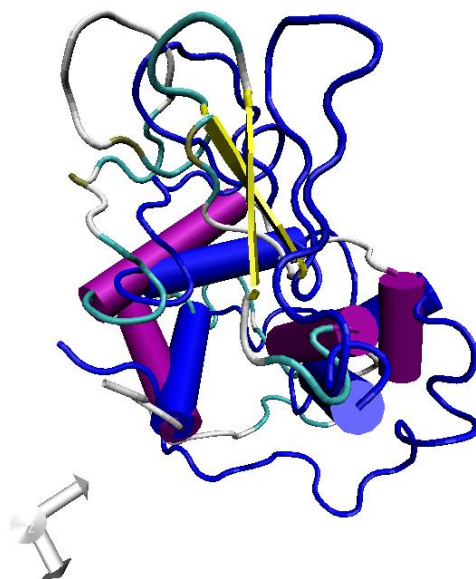
Results and Discussion

Protein conformation

For structure comparison, the root-mean-square deviation (RMSD) was used, which is one of the most commonly used measures. To investigate the effect of the sugars on the conformational changes

of lysozyme, we have calculated the RMSD of the C_{α} atoms of the protein from the NMR structure in Fig. 1a and Fig. 1b and from both the NMR and X-Ray structure in Fig. 1c. The RMSD of the protein calculated as a function of simulated time is shown in Figs 1a, 1b and 1c. The values of RMSD initially increased and reached a plateau after approximately 4 ns in water, 9 ns in the water/fucose solutions, and 12 ns in the water/fructose solutions at high sugar concentrations, while the RMSD increase fluctuates more and is significantly smaller in the water/mannose solution at the high sugar concentrations. At low sugar concentrations the three sugars showed similar behaviors, the RMSD gradually increasing and stabilizing after 18 ns. The presence of sugars at the studied concentrations increases conformational changes of lysozyme, as seen from the increases of the RMSD in Fig. 1a and 1b. In Fig. 1c we show the RMSD for the lysozyme C_{α} atoms from both the NMR and X-ray structure. As one can see there is essentially no difference. The values for all studied systems are given in Table 2.





(e)

Figure 1. Protein root mean square deviations (RMSD) from the NMR structure are represented as a function of simulation time in the lysozyme/water solution and in the different ternary solutions lysozyme/water/sugar. (a) For the system at concentration of 13 wt % of sugar. (b) For the system at concentration of 7 wt % of sugar. (c) Protein RMSD from the NMR and X-Ray structure between 9 and 10 ns for the lysozyme/water solution. (d) Configuration of lysozyme in 13 % mannose solution at 5.8 ns (grey/green) and 17.4 ns (blue), respectively. (e) Configuration of lysozyme in 13 % fucose solution at 5.8 ns (grey/green) and 17.4 ns (blue), respectively.

The conformation of lysozyme in the different solutions has also been characterized by means of its radius of gyration (R_g). The radius of gyration describes the effective size of molecules. The R_g fluctuated and increased more significantly in sugars than in water, indicating the interactions between protein and solvent become more significant. The R_g of protein calculated as a function of simulations time displayed in Figure 2. For the system at high concentrations of sugars the value of radius of gyration is increased compared to the lower concentration of sugars. In the water/fructose solution the value of radius of gyration increased but reached a plateau after approximately 2 ns. The average values of the radius of gyration of lysozyme in the water/sugar solutions at lower concentration are not so different from each other; (table 2, Figure 2b). The RMSD and R_g in monosaccharide solutions increase relative to their value in water (aqueous) solution and they all essentially approach stable values.

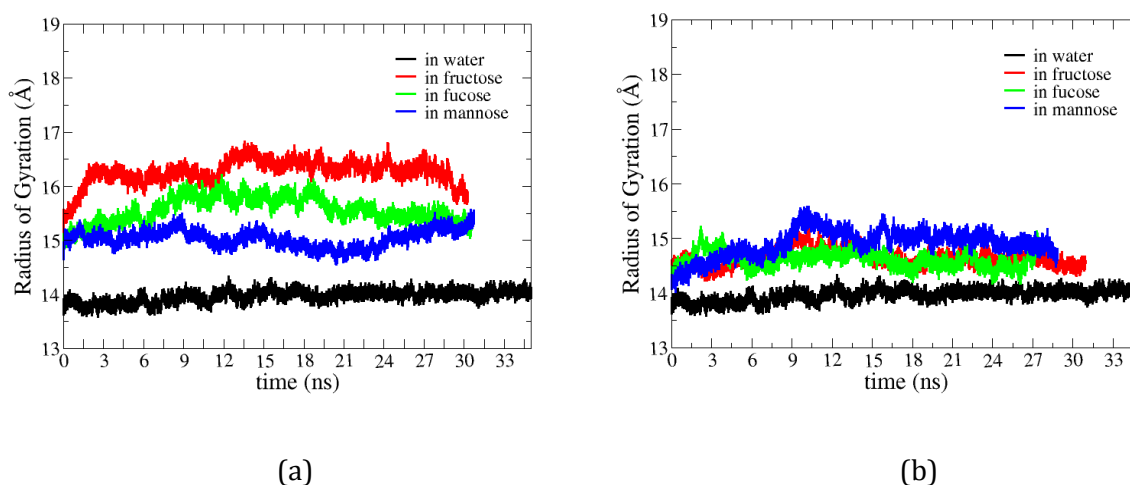


Figure 2. Radius of gyration of atoms as function of simulations time from the lysozyme C_{α} in the lysozyme/pure water solution and in the different ternary solutions lysozyme/water/sugar. (a) For the system at concentration of 13wt % of sugar. (b) For the system at concentration of 7wt % of sugar.

In order to investigate further the effects of various sugars on the conformational changes of lysozyme, the solvent accessible surface area (SASA) has been calculated with a probe radius of 0.14 nm. The SASA of protein was evaluated based on the final 22 ns of each simulation. The SASAs of hydrophobic and hydrophilic residues in the protein have been calculated as well as the total SASAs for the protein in each sugar solution and all are given in given in Table 2.

The SASA of lysozyme is increased in the presence of sugar compared to the SASA of lysozyme in the presence of pure water. This can be because lysozyme in the presence of the sugar exposes more non-polar residues more quickly to the solvent than lysozyme in pure water. The effect of water is clearly shown by an increase in the SASA of the hydrophilic residues and decrease in the SASA of the hydrophobic residues. Hydrophilic residues typically have a large fraction of SASA, but hydrophobic residues have a small fraction of SASA. Differences in the SASA between the two concentrations of sugar have been show in Table 2, the SASA increases when the concentration of sugar increases.

The R_g and SASA of lysozyme in the presence of monosaccharide namely mannose, fructose and fucose are larger, this may stem from the non-negligible interaction of surface residue of lysozyme with sugars.

Protein fluctuations

The influence of osmolytes (sugar) on the internal motions of lysozyme and flexibility of the protein has been investigated by the computation of root mean-square fluctuations (RMSF). Fluctuations of protein backbone atoms around their positions in the original NMR structure have been obtained from the MD trajectories only for the last 22 ns. High RMSF values indicate higher mobility and lower RMSF values indicate restricted mobility. The addition of sugars decreases lysozyme fluctuations and their distributions are not qualitatively the same for all sugars and their concentrations. Figure 3 shows the RMSFs of lysozyme for the different systems by residue number. Significant fluctuations of the protein atoms have been observed for the ternary lysozyme/sugar/water solutions with 13 wt % sugar, a maximum of ~ 1.5 Å, while the maximum for

the 7 wt % sugar solutions was only ~ 1.0 Å. The large fluctuations of the residues are characterized by the RMSF; relatively large fluctuations were observed for the N-terminal and C-terminal tails, but the fluctuation of protein reduced in presence of mannose for the system at concentration of 13 wt % of sugar see Figure 3a.

WT (%)		RMSD (Å)	R _g (Å)	SASA (Å ²)		
Sugar			Residues			
		C _α atoms		All atoms	Hydrophilic	Hydrophobic
0		2.28 (0.21)	14.03 (0.23)	7655 (169)	6132 (179)	1523 (97)
7	Fucose	3.59 (0.61)	14.61 (0.14)	9142 (119)	6976 (81)	2166 (105)
7	Fructose	3.89 (0.72)	14.64 (0.15)	9405 (165)	6824 (113)	2581 (94)
7	Mannose	3.66 (0.76)	14.90 (0.24)	8737 (177)	6587 (99)	2150 (107)
13	Fucose	3.63 (0.59)	15.60 (0.24)	10083 (176)	7023 (95)	3060 (113)
13	Fructose	4.80 (0.54)	16.26 (0.22)	11435 (141)	7968 (119)	3467 (64)
13	Mannose	3.64 (0.36)	15.03 (0.15)	9893 (139)	7119 (86)	2774 (76)
Guinier analysis	Concentration of surfactant (mM)					
Vis/ UV [31]	5.1	13.5/ 13.4				
Vis/ UV [31]	7.9	14.3/ 13.2				
Vis/ UV [31]	12.2	14.7/ 13.4				
	0.0	13.5				
[32]	SANS in D ₂ O	13.3				

Table2. The table presents calculated values of the following parameters describing the structure of lysozyme, in the case of lysozyme/pure water solution and in the different ternary solutions lysozyme/water/sugar: (i) RMSD of the C_α atoms of lysozyme (ii) Radius of gyration of lysozyme, and (iii) SASA of hydrophobic residues, hydrophilic residues of the lysozyme. Standard deviations from mean values are given in parentheses. Experimental R_g values by the Guinier method [31] and small angle neutron scattering (SANS) [32] for comparison.

The protein fluctuations in the presence of mannose (blue) for the 7 wt % of sugar concentration and in pure water (black) are different. In Figure 3 one can see which residues have high RMSFs. These residues correspond to the N-terminus and the first α -domain, and the C-terminus. They are located at the protein-water interface (the exterior of the protein), and therefore are likely to form HBs with the solvent or the sugar molecules. The residues with lower RMSFs, corresponds to α -helices and a loop

located in the core of lysozyme. These residues are not extensively accessible to the solvent (see f_{sa}), and their motions are sterically constrained by the presence of other protein residues.

The RMSF do not reveal clear difference among the three sugars, even if mannose tends to globally reduce slightly more protein fluctuations than fructose and fucose of the ternary lysozyme/water/sugars solution at 7 wt %.

In Figure 4 we present the DELTA (Δ RMSF) calculated using the following equation, $\Delta\text{RMSF} = \text{RMSF}/\text{sugar} - \text{RMSF}/\text{water}$. As one can see in Figure 4, the largest changes in the RMSF are at the N- and C-termini at high sugar concentrations, and that the fluctuations in the mannose solution are significantly smaller than those in the fructose and fucose solutions. At low sugar concentrations, DELTA is negative again at the N- and C-termini for all sugars. What is noteworthy is that for fucose DELTA is negative for all residues.

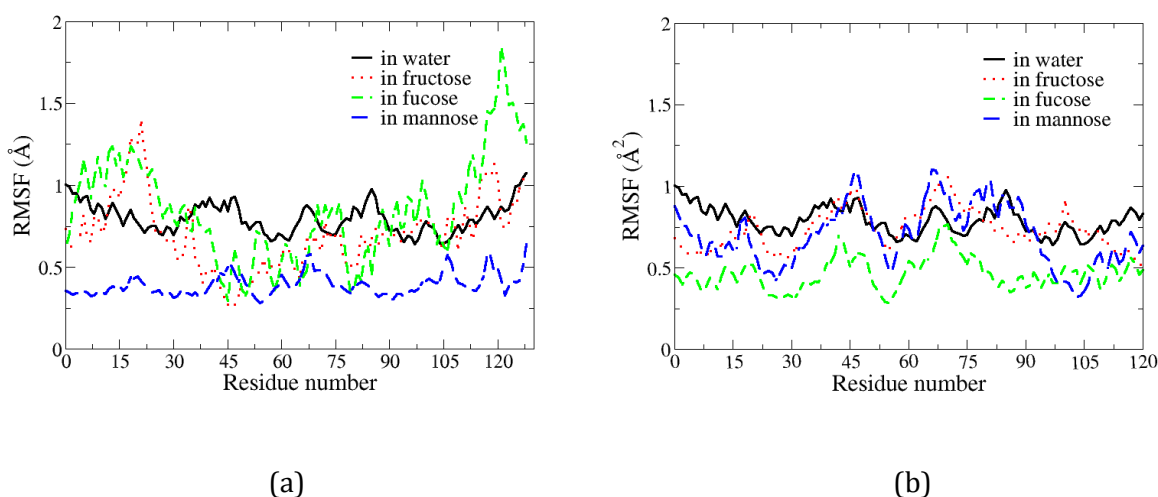


Figure 3. RMSF of atoms from lysozyme in a pure water solution and in the different ternary solutions lysozyme/water/sugar as function of residue-number. (a) For the system at concentration of 13 wt % of sugar. (b) For the system at concentration of 7wt % of sugar. Residues 6-15, 25-36, and 89-100 correspond to the three main α -helices (A, B, and C), while residues 111-114 correspond to α -helix D, and residues 81-84 and 120-123 correspond to 3_{10} helices. Residues 41-60 form the triple stranded antiparallel β -sheet.

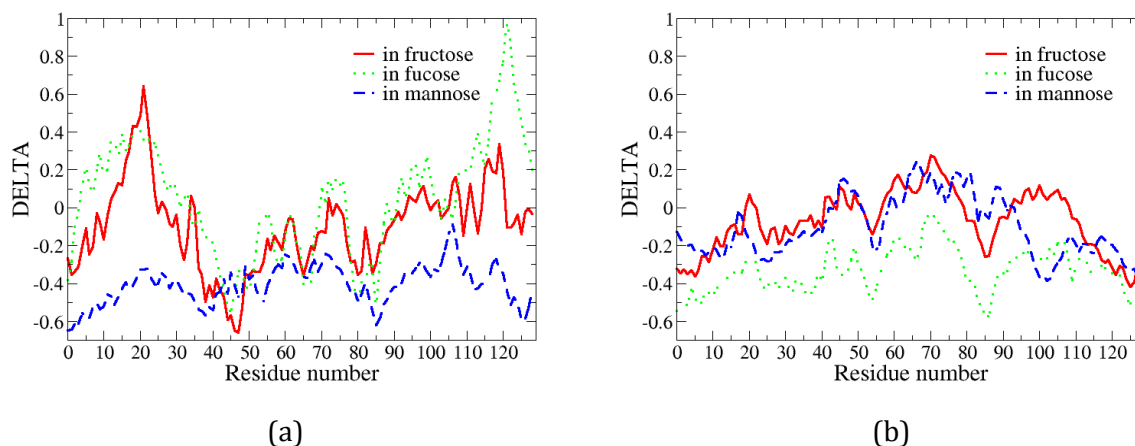


Figure 4. DELTA ($= \Delta \text{RMSF}$) for from lysozyme in a pure water solution and in the different ternary solutions lysozyme/water/sugar as function of residue-number. (a) Δ for the system at concentration 13 wt % of sugar, fructose (red), fucose (green) and mannose (blue). (b) Δ for the system at concentration 7 wt % sugar, fructose (red), fucose (green) and mannose (blue).

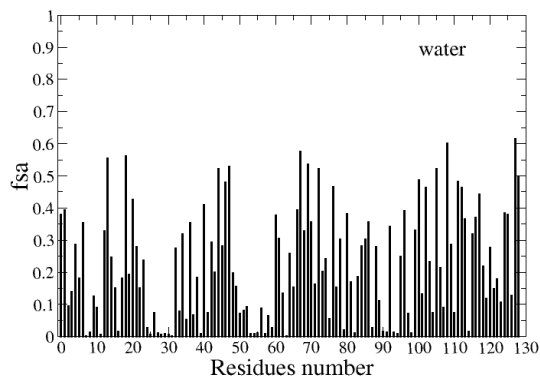
Fractional solvent accessible f_{sa}

The local environment and interaction of protein with the solvent have an effect on the fluctuation of protein residues. Residues from the core should be more densely packed and less accessible to the solvent than residues from the surface and the motions of the core residues are also more sterically constrained [33]. To investigate how the lysozyme residues interact with the solvent or to what degree each residue of lysozyme is exposed to the solvent, we calculated their time averaged fractional solvent accessibilities [34] as $f_{sa} = A_{\text{protein}} / A_{\text{free}}$, where A_{protein} is the SASA of a particular residue in the presence of the other surrounding residues of lysozyme, while A_{free} is the corresponding surface area when no other residues are present, i.e., of the free residue.

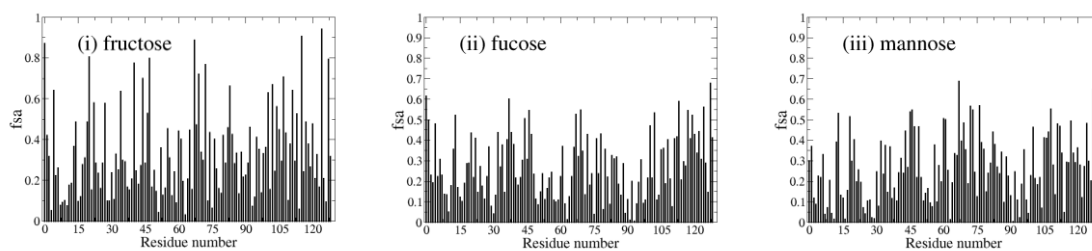
Figure 5 shows the fractional solvent accessible surface areas f_{sa} as a function of the residue for the different systems. Typically, f_{sa} values vary from 0 to ~ 0.61 for lysozyme/pure water and from 0 to ~ 0.7 for the system at 7 wt % concentration of sugar and from 0 to ~ 0.9 for the system at 13 wt % concentration of sugar. The distributions mimic those of RMSFs (see Figure 3), and thus confirm that residues with low solvent accessibilities fluctuate much less than residues exposed to the solvent. The residues for which the f_{sa} values are lower than 0.2 are mainly lysozyme core residues. The residues for which the f_{sa} values are ~ 0.5 are attributed to lysozyme surface residues.

The magnitudes of f_{sa} have increased for lysozyme residues in the presence of sugar compared to those of lysozyme in the presence of pure water. The f_{sa} do not show clear differences for fucose and mannose at the 7 wt % and 13 wt % concentrations and for fructose at the 7 wt % concentration,

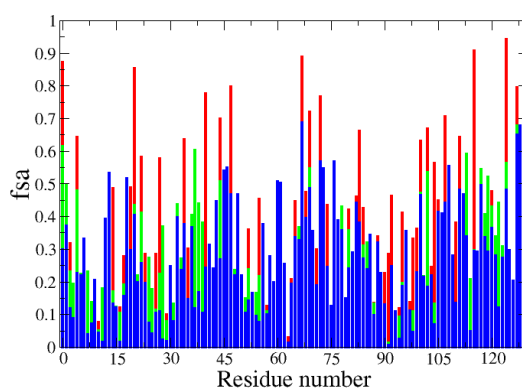
but in fructose the contribution for f_{sa} has increased for 13 wt % the concentration of this sugar as seen in Figure 5b. These results show that the surface residues have larger motions than core residues, because steric limitations established by other protein residues are lower at the surface of the protein.



(a)



(b)



(b)

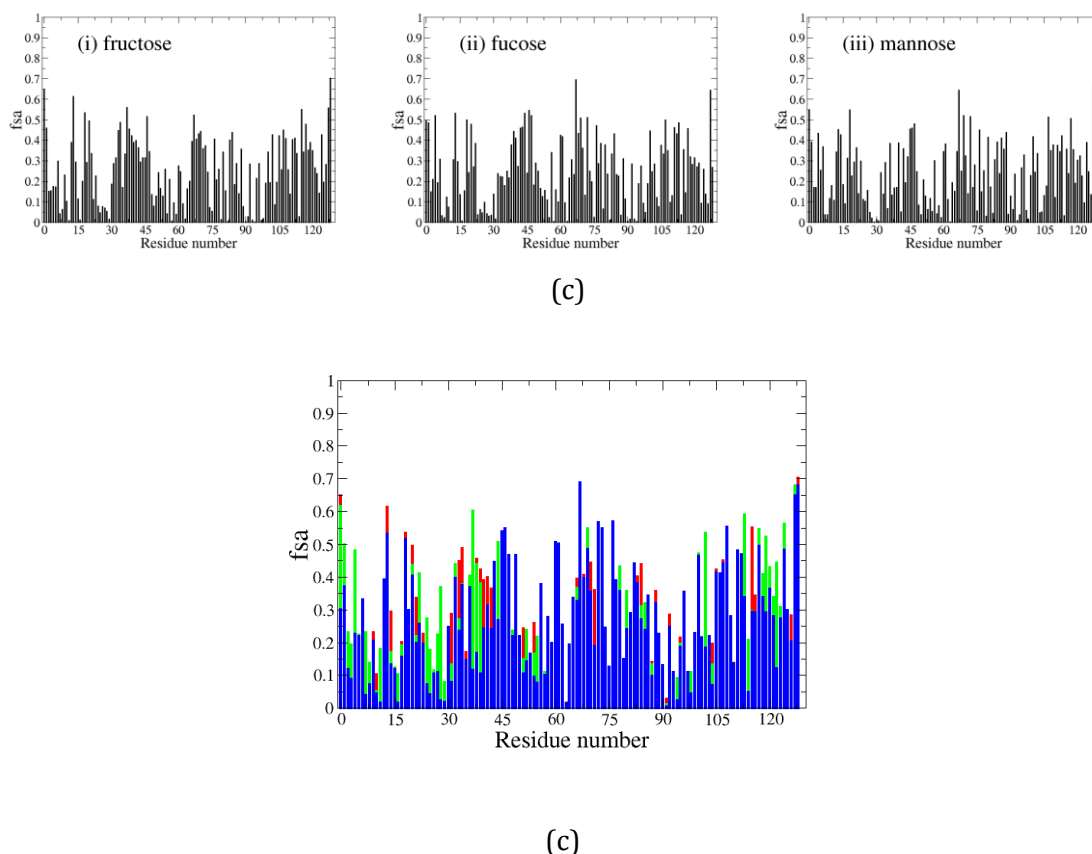


Figure 5. Fractional solvent accessibilities f_{sa} as a function of residue number for the lysozyme in pure water solution and in the different ternary solution (lysozyme/water/sugar).

(a) f_{sa} for the lysozyme in pure water (black). (b) f_{sa} for the system at concentration 13 wt % of sugar, fructose (i) or (red), fucose (ii) or (green) and mannose (iii) or (blue). (c) f_{sa} for the system at concentration 7wt % sugar, with either the same notation (i, ii, iii) in separate plots or color coding (red, green, blue) as in (b) in the single plot.

Hydrogen bonds

The sugars stabilize the protein conformation against dehydration stresses by replacing surrounding water molecule through hydrogen bonds (HBs). We used a cutoff distance of 3.4 Å between the donor and acceptor to define an HB, and a valence angle of less than 120°. A sugar hydroxyl has the capacity to interact with protein both as a hydrogen bond donor and as an acceptor by way of the lone electron pairs. According to the water replacement hypothesis, sugars form HBs with protein by substitution of the hydration water near the surface of the protein and preserve the native structure of the protein [35]. Water is able to form 4HBs, two of them as donors and two of them as acceptors. The hydroxyl group of the sugar forms three HBs, one as a donor and two as acceptors through the lone electron pairs. In the present study, the formation of HBs between lysozyme and osmolytes (sugars) has been analyzed. The number of sugar-protein HBs and the number of water-protein HBs increase with the increasing of sugar concentration. In the investigated concentration range, sugars form between about 5 and 14 % of the total number of protein-solvent HBs, as

indicated in Table 3. This is much fewer than the number of HBs they could form, assuming that water and sugar molecules were equally able to form HBs with lysozyme. Indeed, assuming that water is able to form four HBs (two as donor and two as acceptor) and that each hydroxyl group of the monosaccharides may optimally form three HBs (one as donor and two as acceptor). The proportion of sugar – lysozyme HBs among solvent-lysozyme HBs should be about 2.7 and 5.8 % for the 7 and 13 wt % solutions with mannose and fructose, and 2.2 and 4.7 % for the 7 and 13 wt % solutions with fucose.

The number of monosaccharide-lysozyme HBs and the number of water molecules shared between lysozyme and monosaccharides increase with sugar concentration. Slight differences are observed among the three sugars. Fucose molecules form a lower number of HBs with lysozyme at higher and lower concentration. Indeed, fructose forms more comparable numbers of HBs with lysozyme at lower and higher concentrations. The fucose HBs network maybe less developed than those of mannose and fructose at the studied concentration. Fructose and mannose form essentially the same number of HBs, significantly more than number formed by fucose. Sugar-lysozyme HBs via ring and glycosidic oxygen atoms of sugars are neglected. Although some sugars are preferentially excluded from the surface of lysozyme [36], it appears that in the case of monosaccharides that they may actually preferentially HB with lysozyme. Sugar-protein HBs may actually increase the stabilization of protein, because of the slower relaxation of the sugars-lysozyme HBs as a consequence of the lower mobility of sugars [37,38].

Wt (%)	N_{water}		$n_{\text{HB}} \text{ (W-L)}$	Waters / n_{HB}	$n_{\text{HB}} \text{ (S-L)}$	%HB(S-L)
0	5537		499.72(30.46)	11.08		
7	5217	Fructose	465.3 (60.4)	11.21	44.4 (8.4)	8.7 (1.2)
		Fucose	586.8 (23.5)	8.89	32.9 (4.8)	5.3 (0.8)
		Mannose	503.8 (18.8)	10.36	42.7 (16.8)	7.6 (1.8)
13	4889	Fructose	589.6 (26.8)	8.29	99.59 (10.2)	14.47 (1.6)
		Fucose	545.2 (31.7)	8.97	63.64 (6.8)	10.49 (1.3)
		Mannose	537.1 (22.6)	9.10	90.17 (6.2)	14.38 (0.9)

Table 2. Mean numbers of water-lysozyme and sugar-lysozyme hydrogen bonds, $n_{\text{HB}} \text{ (W-L)}$ and $n_{\text{HB}} \text{ (S-L)}$, respectively, percentage of sugar-lysozyme HBs among water – lysozyme hydrogen bonds (%HB(S-L) = $n_{\text{HB}} \text{ (S-L)} / (n_{\text{HB}} \text{ (S-L)} + n_{\text{HB}} \text{ (W-L)}) \cdot 100\%$). Standard deviations from mean values are given in parentheses.

Preferential hydration

The measurements of preferential hydration providing answer to the question whether co-solvent is stabilize/destabilize protein and preferentially excluded/interacted to the protein surface. The apolar groups of lysozyme do not form HBs with solvent molecules and the number of protein-solvent HBs does not describe exhaustively protein-solvent interaction, then we well characterized the preferential hydration of co-solvent and protein solution. The relative local distribution of water molecules around lysozyme has been characterized and computed as the time-averaged normalized ratio $g_{N,OW} = n_{ow} / (n_{ow} + n_{os})(r) / (N_{OW} / (N_{OW} + N_{OS}))$, where n_{ow} and n_{os} are respectively the local numbers of water oxygen atoms and sugar hydroxyl group oxygen, located at a distance r from the

lysozyme, and N_{ow} and N_{os} denote respectively the total numbers of water oxygen atoms and sugar hydroxyl group oxygen atoms [25]. The sugar molecules preferentially interact with the protein if the ratio is lower than one in the close proximity of the protein surface; conversely, the sugar molecules are preferentially excluded from the protein surface if the ratio is greater than one. This ratio is represented in Figure 6 for the different ternary systems. The normalized ratio value has been lower than one in the close proximity of the protein surface. At the distances larger than ~ 6 Å, there is slight water depletion which results from the presence of sugars at higher concentration, see Figure 6a. From this result it can be concluded that sugar molecules preferentially hydrate with the protein, taking into account that the SASA of lysozyme also increases in the sugar solutions, that is, the total number of HBs is also larger. Fucose preferentially hydrates lysozyme more than do mannose and fructose. These maybe fucose hydroxyl oxygens were indeed systematically more excluded from the apolar groups of lysozyme than mannose and fructose. The preferential hydration may be dependent on the concentration of sugar. Lysozyme is clearly more preferentially hydrated when the sugar concentration increased see Figure 6a. Lerbret *et al.* [33] have shown that lysozyme has been more hydrated with increasing the concentration of sugars (trehalose, maltose, and sucrose) and it has been slightly difference of lysozyme preferential hydration between at the concentration 37 wt % and 60 wt % of sugars. Cotton *et al.* [25] and Beck *et al.* [39] have also shown that carboxy-myoglobin has been preferentially hydrated in presence of sugar at concentration of 89 wt % of sugar.

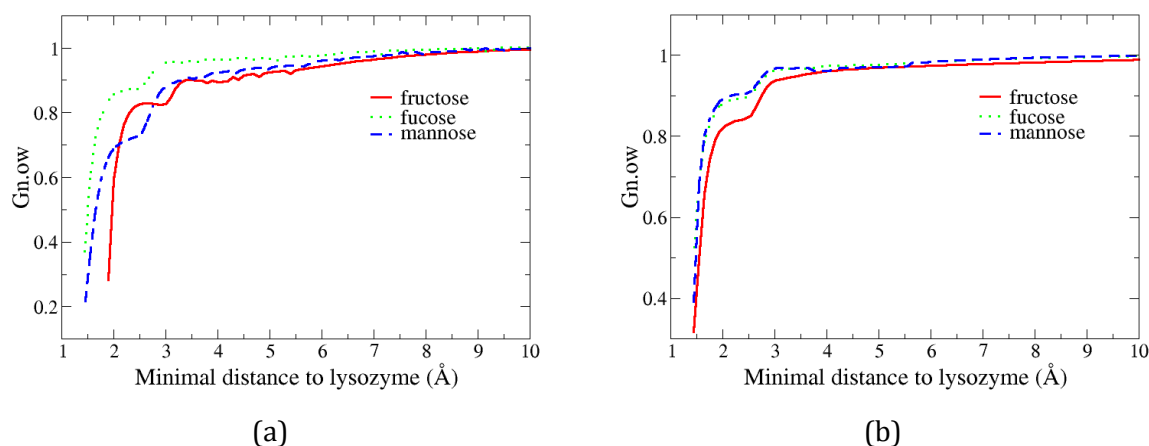


Figure 6. Normalized fraction of water oxygen atoms as function of the minimal distance to closest atom of lysozyme. (a) Normalized fraction of water oxygen atoms as function of the minimal distance to the lysozyme for the concentration 13 wt % of fructose (red), fucose (green) and mannose (blue). (b) for the system at concentration 7 wt % of sugar.

Solvent Mobility at the interface with proteins

The presence of protein in solvent has effect on mobility of the surrounding solvent. It is convenient to consider a description of the solvent distribution around the protein in the form of density as has been shown and described in Makarov *et al.* [39,44]. There is a region inside the protein where the solvent does not easily penetrate or where the probability of finding it is very low (solvent free region or region between

0-2.4 Å for water oxygen atom and 0-2.3 Å for sugar oxygen atom with respect to water oxygen atom), see Figure 7. Outside of the hydrophobic region there is a transitional region (region of interpenetration of protein and solvent or region of 2.4 Å for water oxygen atom and 2.3 Å for sugar oxygen atom with respect to water oxygen atom) that can be seen in Figure 7 that can be characterized by a significant degree of interpenetration of protein and solvent. The value of solvent density in this region is smoothly increasing as one moves away from the protein and into the solution. It has been proposed [40] that solvent penetration provides a significant contribution to the high apparent polarizability inside the protein. A large number of local density maxima are found in the (region 2.4- 3.2 Å for water oxygen atom and 2.3-3.2 Å for sugar oxygen atom with respect to sugar oxygen atom) in Figure 7. The density value over the first hydration shell is higher than that of the bulk solvent as evidenced in simulation [41] and experimental observations [42]. The local peaks are often referred to as hydration sites. While sites are often too close to each other to be interpreted as individual solvent molecules. The mobility of buried interfacial water and solvent is often severely restricted. Those water and solvent molecules that are more exposed to bulk have much shorter residence times than that in the grooves, clefts and buried inside the protein [43,44]. The chemical differences between hydration site as polarity of the surrounding residues or the ability of the hydration water to hydrogen bond to the protein at the given site, only affect the residence times of water in the less accessible locations [45]. Water in the nonpolar atomic sites has a short residence time irrespective of the solvent accessibility [46]. Further into the bulk of solvent (at a distance of roughly 4-7.4 Å for water oxygen atom) finds only weak maxima corresponded to the second hydration shell. The second shell for fructose at distance 3.1- 4.5 Å and for fucose at distance 3.3-5.9 Å and for mannose at distance 3.3-5.6 Å have been seen. The third shell have been seen at distance between 5.6-8.2 Å for fructose 6.0-8.2 Å and for fucose, and 5.7-8.4 Å for mannose. At distances greater than 9 Å from the protein, solvation shells cannot be discerned and this is a remote region, where no maxima are found, but diffusion rate is perturbed.

For the system having a 7 wt % concentration of sugars, the first coordination shell at distance 2.4-3.3 Å have been seen for water oxygen atom with respect to sugar oxygen atoms. The second shells for fructose at distance between 3.2-5.6 Å, for fucose between 3.3-6.0 Å and for mannose between 3.2-5.7 Å have been seen. The third shell for fructose between 5.8-8.8 Å, for fucose between 6.0-8.2 Å and for mannose between 5.8-8.2 Å have been seen.

Radial Distribution Function

The RDF was used to describe the distribution of water and sugar as the function of distance from protein surface. The RDF of water oxygen-oxygen atom and monosaccharaides oxygen atom respect to water oxygen atom were calculated for all seven simulations. It is convenient to consider a distribution of solvent in the form of RDF and shown in Figure 7 for the system having 13 wt % and 7 wt % concentrations of sugars.

For the lysozyme systems with 13 and 7 wt % sugars and in pure water, the RDFs of sugars oxygen atoms respect to water oxygen atoms are represented in Figure 7, while in Figure 8 the RDFs for the lysozyme systems with 13 and 7 wt % sugars are given (to show more details in the solvent shells). The total exclusion of solvent and co-solvent at distances less than 2.3 Å for sugar oxygen atoms with respect to water oxygen atom can be seen, and less than 2.4 Å for water oxygen atoms.

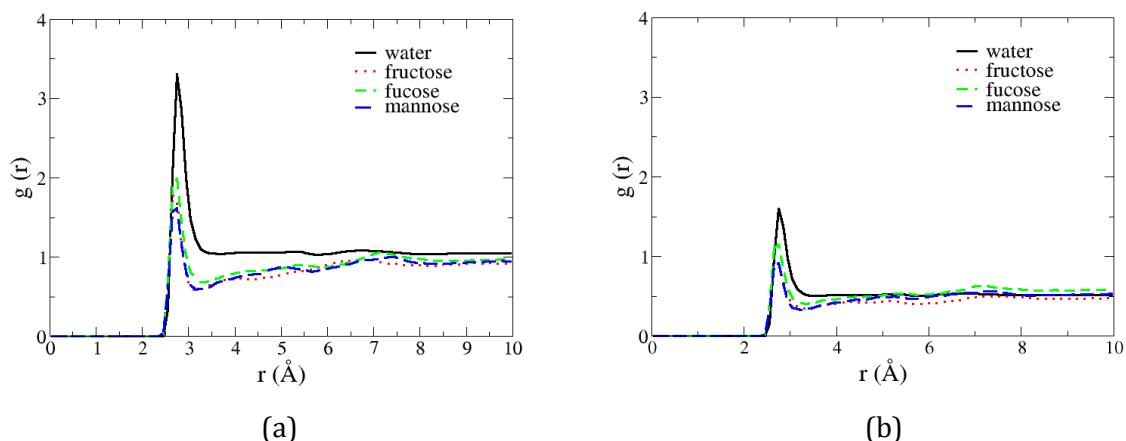


Figure 7. RDF for the system of lysozyme in 13 wt % and 7 wt % sugar and pure water. (a) RDF of water oxygen atoms with respect to water oxygen atoms (black) and water oxygen atoms with respect to sugar oxygen atoms (fructose (red), fucose (green) and mannose (blue)) for the system with 7 % wt sugar, (b) for the system with 7 wt % sugar.

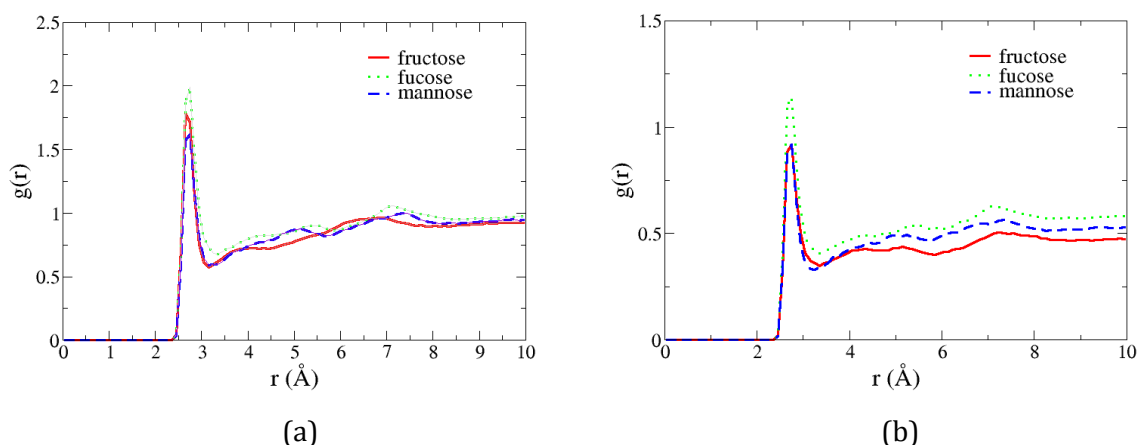


Figure 8. RDF for the systems of lysozyme in 13 wt % and 7 wt % sugar. (a) RDF of water oxygen atoms with respect to sugar oxygen atoms (fructose (red), fucose (green) and mannose (blue)) for the system with 13 % wt sugar, (b) for the system with 7 wt % sugar.

The inner hydration shell shows molecular orientations very dependent on the local character of the protein surface (either nonpolar, or positively or negatively charged), whereas the second shell extends continuously and prefers orientations which optimizes electric dipole-electric dipole interactions in the first few Å of this shell when the closest protein atoms are either positively or negatively charged and hence possess local dipole moments. The first maximum in the radial distance appears at 2.73 Å for the water oxygens atom and at 2.76 Å for sugar oxygen atoms. With respect to water oxygen atom, these maxima represent the centers of the inner hydration shell layers formed by the hydrogen bonds between the solvent and polar atoms of lysozyme. The second maximum is due to the outer hydration shell layer formed by water

hydrogen bonds and sugar hydrogen bonds with lysozyme, and van der Waals interactions between solvent and nonpolar atoms of the protein.

The geometries and positions of the peaks in the RDFs of water oxygen atoms and the sugar oxygen atoms with respect to water oxygen atoms around the protein for high and lower concentration of sugars are different. The RDFs peaks for low concentration are smaller compared to the high concentrations. Here the sugar oxygen atoms appear to be less exposed to the protein at the lower sugar concentration.

Conclusions

This article reports the results from molecular dynamics simulations of lysozyme/monosaccharide/water solutions for intermediate concentrations of monosaccharide (7 and 13 wt % on a protein-free basis). Several parameters of lysozyme like its RMSD, radius of gyration and SASA indicate that its conformation in the presence of sugars remain similar to that in pure water. Fluctuation of lysozyme as seen from RMSF is reduced in the presence of monosaccharide. This reduction was shown to be not homogeneous throughout the protein and follows the fractional solvent accessibility of lysozyme residues. The analyses of the interactions of sugars with lysozyme via HBs suggest that lysozyme is preferentially hydrated. Fucose was found more preferentially excluded from the protein surface in high concentration of sugar (13 wt %) than fructose and mannose. As a consequence, its concentration in the bulk may be slightly larger than that of fructose and mannose. When comparing the three sugars the slight differences were not observed. The number of HB was found smaller in fucose solutions in comparison with fructose and mannose. This could be interpreted by the larger number of intermolecular HBs of fructose. The HBs of fructose were found to be larger than that of fucose and mannose was found to have the largest SASA. The interaction between the solvent and protein was also represented by radial distribution function. The RDF displays the characteristic of solvent around the protein. The hydration shell is very dependent on the local character of the protein surface. From the maxima of the RDF indicated that hydration water is denser and narrowly defined, due to the stronger interaction induced by the protein.

Acknowledgements

We would like to thank Morten Jensen, Per Jr. Greisen, and Rasmus Wedberg for scientific discussions, comments and suggestions on how to improve this work. In addition, we would like to thank the University of Southern Denmark for providing computational resources which were used to perform the molecular dynamics simulations presented in this work. We would like to thank the University of Illinois at Urbana-Champaign (UIUC) for providing both NAMD and VMD which were the programs used for the simulations and subsequent analysis.

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